Technical Report

DEVELOPMENT OF A NOVEL TECHNOLOGY FOR THE MANIPULATION OF FISH REPRODUCTIVE CYCLES; CONTROLLED RELEASE OF GONADOTROPIN RELEASING HORMONES

Robert Langer and Yonathan Zohar

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Development of a Novel Technology for the Manipulation of Fish Reproductive Cycles: Controlled Release of Gonadotropin Releasing hormones.

Robert Langer and Yonathan Zohar, Massachusetts Institute of Technology, Cambridge, MA, USA

1. INTRODUCTION

Our research during the first 2 years of the MIT SeaGrant project was focused on the following areas:

1. Introduction of superactive GnRH analogs (GnRHa) into two polymeric controlled release delivery systems: Ethylene vinyl acetate copolymer (EVAC) and polylactic acid-polyglycolic acid copolymer (PLGA).

2. Characterization of the *in-vitro* release pattern of the GnRHa from the above delivery systems.

3. Characterization of the *in-vivo* release pattern of the GnRHa from the above delivery systems in seabream (*Sparus aurata*) and in Pacific coho salmon (*Oncorhynchus kisutch*) and of their relationships to gonadotropin secretion.

4. Study of the effect of the above delivery systems on ovulation and spawning in seabream, rainbow trout (*Salmo gairdneri*), Atlantic salmon (*Salmo salar*) and Pacific coho salmon.

2. DESCRIPTION OF RESEARCH ACTIVITIES

2.1. Introduction of superactive GnRH analogs into polymeric controlled release delivery systems.

To start our studies, we selected two polymeric controlled release devices which had been previously used in mammalian studies for the controlled administration of peptides and protein. The first was a controlled diffusion, non biodegradable system made of the ethylene vinyl acetate copolymer (EVAC) and the second was a

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biodegradable system made of the biodegradable copolymer of the polylactic and polyglycolic acids (PLGA).

2.1.1. EVAC systems: We made EVAC devices containing one of the two GnRH analogs which have been found to be the most potent in inducing gonadotropin release and ovulation in both the seabream and salmonids. The devices contained either the mammalian [D-Ala^6,Pro^9-NET]-LHRH (mGnRHa) or the piscine [D-Arg^6,Pro^9-NET]-salmon-GnRH (sGnRHa). The EVAC was extensively washed in ethanol and then dissolved in methylene chloride to obtain a 15% solution. 15 ml of this solution was vigorously mixed with a lyophilized mixture of BSA-Inulin-GnRHa to obtain a 40% protein load and GnRHa doses of 25, 75, or 150 μg/device. A homogeneous suspension of the protein in the polymer was obtained by the use of an ultrasonic probe The mixture was then poured into a 5x5 cm mold leveled on a block of dry ice. The EVAC-GnRHa slab was then placed in a freezer at -30°C for 48 hours to allow evaporation of the majority of the methylene chloride. The rest of the solvent was then evaporated by placing the slab in a vacuum oven kept at room temperature. 3 mm discs were cut out from the slabs and individually weighed. In order to obtain coated single aperture devices, each disc was mounted on the tip of a 23 gauge syringe needle and briefly dipped into a 15% EVAC solution.

2.1.2. PLGA systems: We made PLGA microspheres containing the mammalian [D-Ala^6,Pro^9-NET]-LHRH. Three different compositions of PLGA were used: 75:10 (the abbreviation indicates a copolymer consisting of 75% poly lactic acid and 25% poly glycolic acid with a M.W. of 10,000 D), 75:05, and 50:05. The PLGA powder was dissolved in methylene chloride and poured into a solution containing gelatin and the mGnRHa to obtain a final 2% load of gelatin and a 3% load of mGnRHa. The mixture was vigorously mixed using an ultrasonic probe. The emulsion obtained was poured into a 1% polyvinyl alcohol (PVA) solution saturated with methylene chloride and mixed vigorously for 3 hours. This last step results in the formation of the PLGA-GnRHa microspheres. The microspheres were then poured through a 180 μm filter, rinsed with distilled water and lyophilized.

2.2. Characterization of the *in-vitro* release rates of the GnRHa from the polymeric delivery systems.

2.2.1. Methods:

Individual EVAC devices containing different doses of the mGnRHa (25, 75, and 150 µg), coated and uncoated were introduced into 20 ml scintillation vials. They were placed in 5 ml of 1/30 M phosphate buffer containing 0.02% tween 80 at pH 7.0. 2.5 mg microspheres representing the different PLGA preparations (75:10, 75:05, and 50:05), each containing 75 µg mGnRHa/2.5 mg microspheres, were introduced into the same buffer under the same conditions. The scintillation vials were placed in a water bath maintained at 10ºC (the temperature at which salmon broodfish are usually held) and agitated constantly. Initially at 6 and 24 hours and then at 2 to 3 days intervals we sampled either the entire buffer content of the vials (for the EVAC devices) or 1 ml of it (for the PLGA devices). These volumes were replaced by fresh buffer. The buffer samples were immediately frozen and kept at -30ºC for the measurement of their mGnRHa content by specific and sensitive radioimmunoassay. The experiment lasted 37 days.

2.2.2 Radioimmunoassays for GnRH analogs:

A specific homologous radioimmunoassay (RIA) for [D-Ala^6,Pro^9-NET]-LHRH was used for the determination of its levels in serum. 50 µl of a diluted plasma, medium, or standard sample were incubated with 50 µl of rabbit antiserum against [D-Ala^6,Pro^9-NET]-LHRH in a final volume of 500 µl for 24 hours at 4ºC. Incubation was done in 0.01 M phosphate saline buffer pH 7.6 containing 0.2% of BSA. After 24 h, 50 µl of radiolabelled I^{125}-[D-Ala^6,Pro^9-NET]-LHRH was added to all tubes and the incubation was continued at the same conditions for another 24 hours. At the end of this incubation the bound fraction of the [D-Ala^6,Pro^9-NET]-LHRH was precipitated using a second antibody, raised against rabbit γ globulins. The precipitate was counted in a γ radioactivity counter. The plasma levels of the GnRHa were calculated after a log-logit linearization of the standard curve. The sensitivity of the RIA was 0.02 ng/ml and its precision (intraassay variability) was 3.2%. A very similar RIA was used for measuring levels of of D-Trp^6-LHRH, when devices containing this analog were tested. The sensitivity of the latter RIA was 0.05 ng/ml and its precision was 3.2 %.
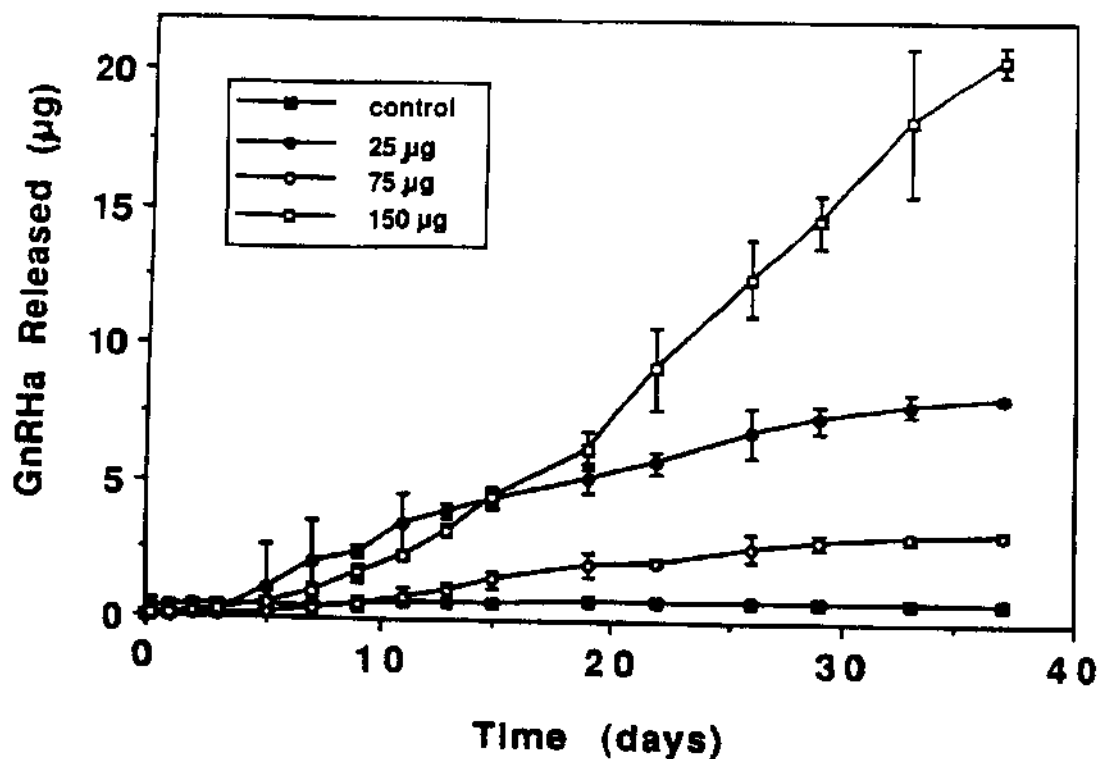
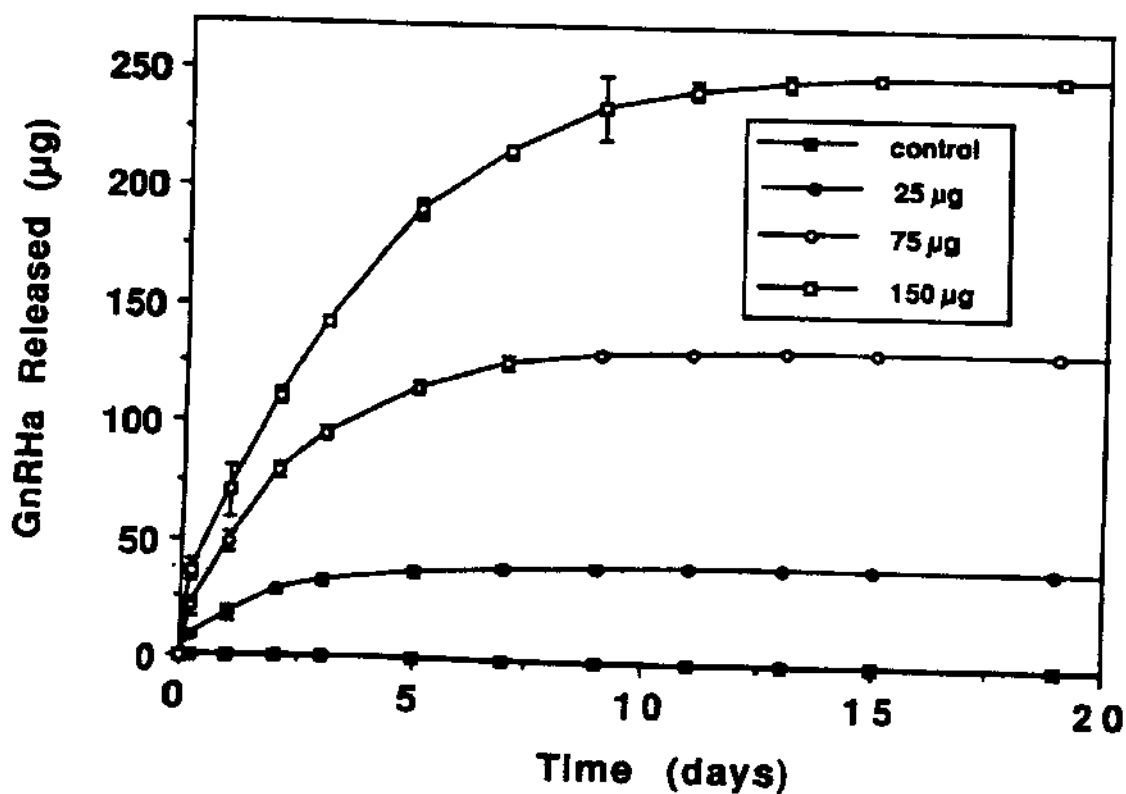Figure 1: Cumulative release of mGnRHa from coated EVAC devices.

Figure 2: Cumulative release of mGnRHa from uncoated EVAC devices.

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2.2.3. Results:

Release patterns of mGnRHa from coated and uncoated EVAC devices are shown in Figs. 1 and 2, respectively. The coated devices released the mGnRHa relatively slowly. Significant mGnRHa release was seen only 7 days after the beginning of the experiment (Fig 1). From that time on the devices steadily released the peptide. However, by the end of the experiment (day 37), the coated EVAC devices had released only a small portion of their entire mGnRHa content. The total amount of mGnRHa/device, as measured by RIA, was estimated from data obtained from uncoated devices (Fig. 2), which released their entire content. On the basis of this estimation, we calculated that the coated devices released only 20%, 2.4% and 8.2% of their content (for the 25, 75 and 150 µg devices, respectively) after 37 days of incubation. While the 150 µg coated devices released their mGnRHa faster than the other coated devices, the 25 µg devices released the peptide faster than the 75 µg devices.

The release pattern of the mGnRHa from the uncoated devices was found to be completely different (Fig. 2). The uncoated devices began to release the hormone as soon as they were introduced into the buffer. A significant release of mGnRHa was observed 6 hours after the initiation of the experiment. From that time on, the uncoated devices released the peptide at constant rates for several days, until the release rates were slowed down just prior to the depletion of the hormone. A very clear dose related release of the mGnRHa from the uncoated devices was observed. The 25 µg devices released their entire content over a period of 5-7 days, the 75 µg devices were depleted by 9 days and the 150 µg devices completed their release by day 15.

Conclusions: The *in vitro* release pattern of mGnRHa from EVAC devices at 10°C was characterized. Coated EVAC devices released the mGnRHa very slowly. In fact, the majority of the hormone was not released from the device after 37 days of incubation. However, the uncoated EVAC devices were found to efficiently release their total dose of mGnRHa at constant rates over a period of 5-15 days. Therefore, these devices might be very efficient in stimulating both immediate and sustained GtH secretion and in inducing ovulation and spawning in different fish species. The *in-vivo* potency of the different EVAC devices was tested in seabream and salmonid species (see below). We are presently continuing to characterize the *in vitro* release pattern of mGnRHa from different polymeric devices at both 10 and 20°C.

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2.3. Characterization of the *in-vivo* release rates of the GnRHa from the delivery systems and its effects on gonadotropin secretion, ovulation, and spawning.

2.3.1. Seabream:

2.3.1.1. Effect of different polymeric GnRHa containing delivery systems on gonadotropin secretion.

Methods: Female seabream undergoing final stages of vitellogenesis were selected for the experiment, and divided into 5 groups of 7-10 each. Two groups were treated with a commercially available medical preparation of PLGA 50:05 containing the mammalian GnRH analogs ICI-118,630 (for one group) and D-Trp6-LHRH (for another group). Two other groups were treated with our non-coated EVAC discs, containing 150 µg of either the mGnRHa (for one group) or the sGnRHa (for another group). The control group received EVAC devices which did not contain the hormone.

The females were bled before the administration of the devices and at 1, 2, 3, 4, 7, 9, 11, and 13 days later. Their plasma was then separated and immediately frozen. The levels of the gonadotropin (GtH) in the plasma were determined by a specific homologous RIA for seabream GtH, developed in Dr. Zohar's laboratory. Also, at each sampling time we removed a few oocytes from the ovaries of the experimental females, to determine the stage of their development.

Results: GtH levels in the plasma of the treated females are shown in Fig. 3. Circulating GtH levels in the control females remained low and unchanged throughout the experimental period, and their vitellogenic oocytes underwent rapid atresia. However, the controlled administration of the GnRH analogs via all tested delivery systems, biodegradable or not, induced an initial burst of gonadotropin secretion, followed by constantly elevated rates of GtH secretion for 9-13 days. In all the females receiving the hormonal devices, oocytes started to ovulate within 3 days of the beginning of the experiment, and 80-100% of the females continued to ovulate at 24 hours intervals.
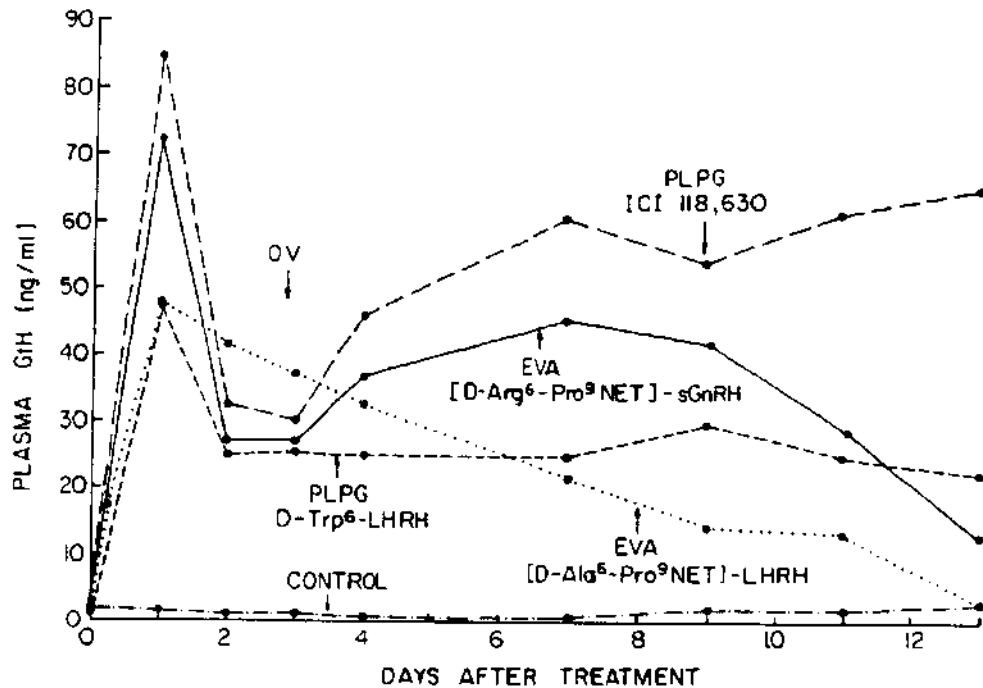
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Figure 3: Plasma GtH levels in the female seabream before and after treatment with various controlled release polymeric devices containing GnRHa. PLPG= polylactic polyglycolic acid copolymer, EVAC= ethylene vinyl acetate copolymer, OV= ovulation

Conclusions: The two tested delivery systems, containing the different GnRH analogs, were found to be highly effective in stimulating intensive GtH secretion from the pituitary of the treated females for at least 9-13 days and in inducing daily cycles of ovulations (which are induced in only 25-30% of females receiving a single injection of GnRHa). Therefore, we continued to study the use of EVAC and PLGA as controlled GnRHa delivery systems for the induction of ovulation and spawning in seabream. Since the mammalian GnRH analogs were found to be as effective as the fish GnRH analog, and since they are less expensive, we focused our further research on the study of devices containing the mammalian analogs. We thus developed specific RIAs for their quantification (described above).

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2.3.1.2. *In vivo* mGnRHa release from EVAC and its effect on GtH secretion and ovulation.

Methods: The experiment was carried out at NCM, Eilat. 10 female seabream with oocytes undergoing final stages of vitellogenesis were treated with uncoated EVAC devices containing 150 μg of the mGnRHa. A similar group of females received EVAC devices with no hormone. Females were bled before and at 1, 2, 6, 9, 13, 16, 27, and 34 days after the administration of the devices. Their plasma was then separated and immediately frozen. The levels of the mGnRHa and of the gonadotropin (GtH) in the plasma were determined by specific and homologous RIAs as described above. Also, at each sampling time we removed a few oocytes from the ovaries of the experimental females, to determine the stage of their development.
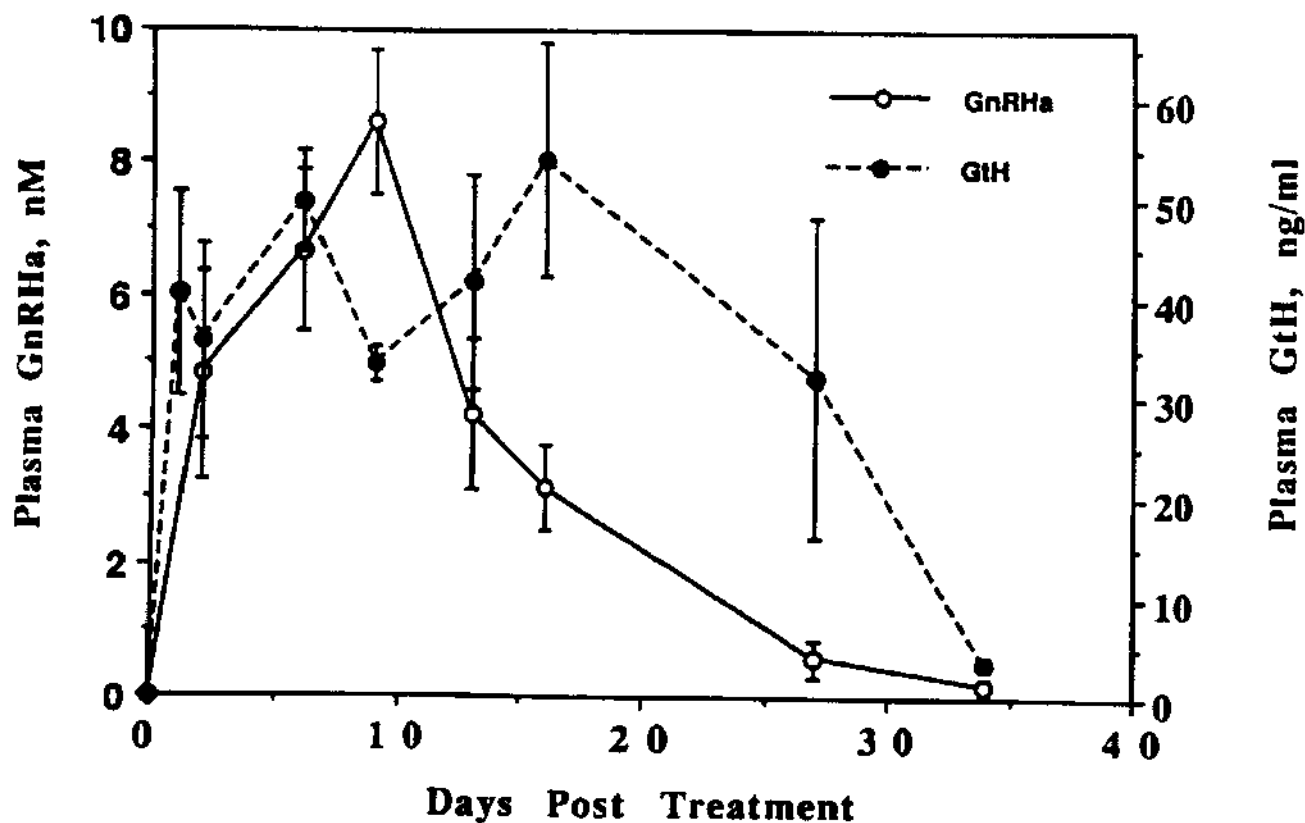

Figure 4: Plasma levels of GnRHa and of GtH in female seabream before and after their implantation with EVAC-GnRHa devices

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Results: No mGnRHa was found in the plasma of the control females, and their circulating GtH levels were low (<0.5 ng/ml) and constant, while their vitellogenic eggs underwent rapid atresia. The plasma levels of mGnRHa and GtH in females receiving the EVAC devices containing the hormone are shown in Fig. 4. The EVAC devices started to release the GnRHa as soon as they were implanted, plasma GnRHa levels being elevated (around 5 nM) by 48 hours. The levels of the GnRHa in the plasma continued to increase and peaked by 9 days post implantation. Later on, GnRHa levels in the plasma decreased steadily, to reach low values by day 27. GtH secretion from the pituitary was highly stimulated as soon as 24 hours after the administration of the EVAC-GnRHa devices (Fig. 4). Circulating GtH levels remained very elevated (at 35-55 ng/ml) for 16 days, and then started to decrease, in parallel to the decline in the levels of the GnRHa, to reach levels comparable to those found in the control females by 34 days. All the females that received the EVAC devices containing the GnRHa ovulated within 72 hours after the implantation and continued to ovulate daily for a period of 20-30 days.

Conclusions: Uncoated EVAC devices intensively released the GnRHa in seabream for a period of about 16 days. This *in-vivo* release is in close agreement with the *in-vitro* release observed for similar devices (Fig. 2), which lasted for about 15 days. The released GnRHa stimulated sustained and intensive GtH secretion, which lasted for 27-34 days. This GtH elevation in the blood in turn induced daily cycles of ovulation for prolonged periods. Our further studies indicated that much lower GnRHa levels than those released from the present EVAC devices are sufficient to induce similar amplitudes of GtH secretion and daily ovulations and spawning (see below). As a consequence we carried out more experiments testing the effect of EVAC devices containing lower doses of GnRHa (25 and 75 µg) on GtH secretion, ovulation and spawning in the seabream. These experiments were completed, and are presently being analyzed.

2.3.1.2. *In vivo* mGnRHa release from PLGA and its effect on GtH secretion and ovulation.

a. PLGA 5005.

Methods: 11 female seabream undergoing the final stages of vitellogenesis were injected with PLGA microspheres containing mGnRHa (D-Trp[6]-LHRH) at the dose of 150 μg/kg body weight. The preparation used was a commercially available medical preparation made of the PLGA 50:05. The control group was injected with the vehicle solution of the PLGA. Blood samples were taken before the administration of the PLGA, at 24 and 48 hours after the injections, and then at 1 week intervals until 80 days post-treatment. Their plasma was then separated and immediately frozen. The levels of the mGnRHa and of the gonadotropin (GtH) in the plasma were determined by specific and homologous RIAs as described above. Also, at each sampling time we removed a few oocytes from the ovaries of the experimental females, to determine the stage of their development.
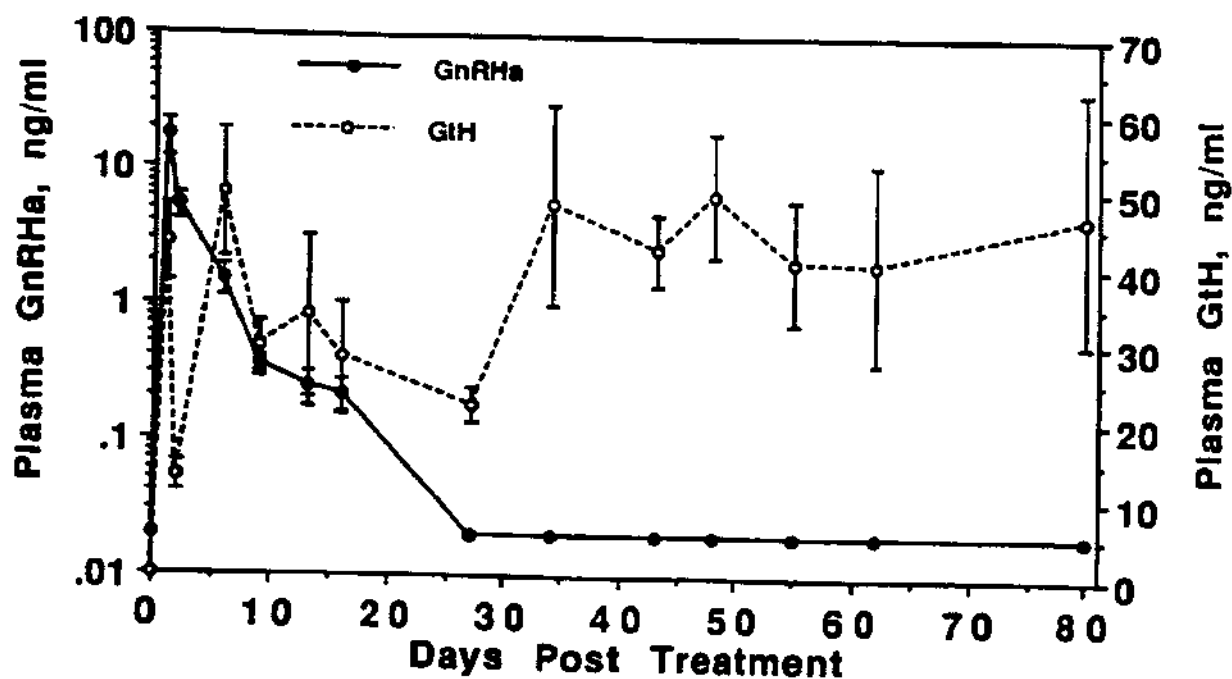

Figure 5: Plasma levels of GnRHa and of GtH in female seabream before and after their treatment with 50:05 PLGA-GnRHa.

Results: The plasma levels of GnRHa and of GtH at different times after the administration of the PLGA-GnRHa microspheres are shown in Fig. 5. The release of the GnRHa from the PLGA 50:05 was found to be characterized by a rapid and very intensive initial burst, and by slower rates later on (Fig. 5). Plasma GnRHa levels peaked as soon as 24 hours after the administration of the microspheres, and decreased

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continuously thereafter. No GnRHa was detectable by our RIA after day 16. GtH secretion in the treated females was stimulated within 24 hours after the administration of the PLGA-GnRHa microspheres (Fig. 5). Circulating levels of GtH fluctuated thereafter, but, despite the non-detectable GnRHa levels, remained elevated (15-55 ng/ml) throughout the entire experimental period of 80 days. All the females treated with the PLGA-GnRHa microspheres ovulated within 72 hours after the injection, and continued to ovulate daily for a period of 20-60 days.

Conclusions: The microspheres made of the biodegradable PLGA copolymer 50:05 released most of their GnRHa content during a period of 16 days. Later on a very slight release may occur, which results in very low circulating levels of GnRHa (below 20 pg/ml), these levels are undetectable by our RIA. However, in spite of this pattern of GnRHa release, GtH secretion continued to be stimulated for a period of 80 days, and the treated females ovulated daily for up to 60 days. Plasma GtH levels remained very high during this period, and did not decrease in parallel to the decline in plasma GnRHa, as observed in females treated with the either the EVAC-GnRHa or the 75:10 PLGA devices (see Figs. 4 and 6). We are presently trying to understand this phenomenon by treating female seabream with controlled release delivery systems containing decreasing dosages of GnRHa.

The PLGA-GnRHa preparation tested in this experiment was a commercially available one. We have recently prepared microspheres made of different PLGA copolymers, and are presently testing their efficiency in releasing GnRHa both *in vitro* and *in vivo*, and in inducing GtH secretion and ovulation in seabream and salmon species. Some of these data is shown and discussed in the next paragraph.

b. PLGA 7510

Methods: 7 female seabream undergoing the final stages of vitellogenesis were injected with PLGA microspheres containing 75 µg/kg body weight mGnRHa ([D-Ala^6,Pro^9-NET]-LHRH) . The microspheres prepared by us as described in section 2.1.2. Seven control females were injected with the vehicle solution of the PLGA. Blood samples were taken before the administration of the PLGA and at 1, 3, 6, 10, 14, 21, and 28 days after the injections. Their plasma was then separated and immediately frozen. The levels of the mGnRHa and of the gonadotropin (GtH) in the plasma were

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determined by specific and homologous RIAs as described above. Also, at each sampling time we removed a few oocytes from the ovaries of the experimental females, to determine the stage of their development.
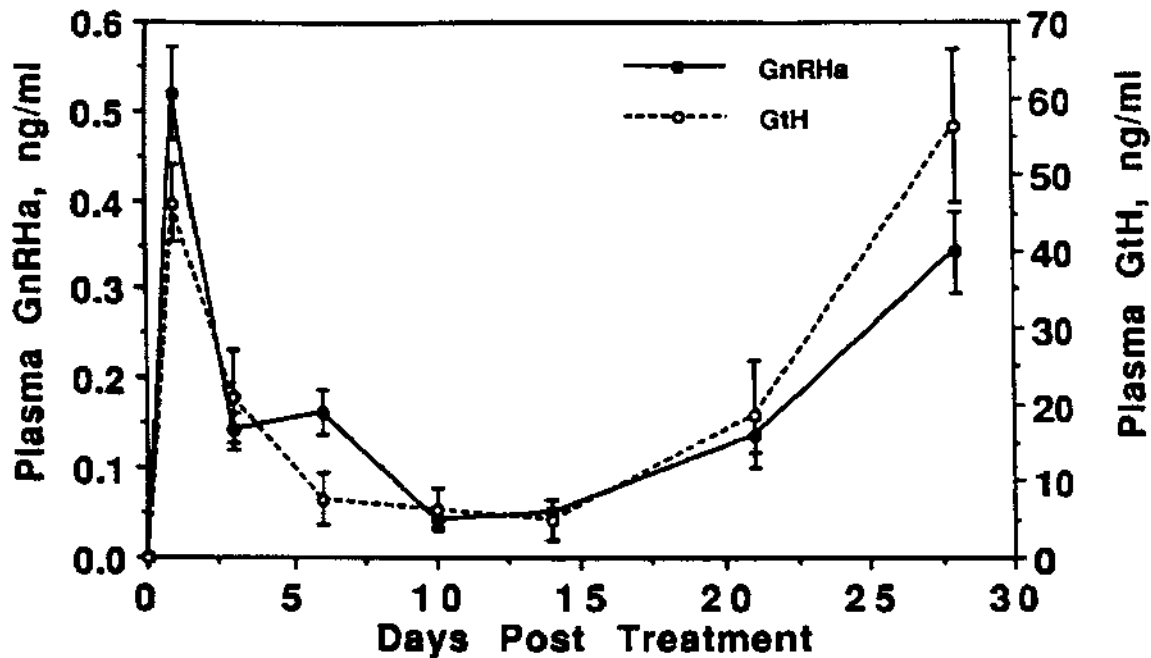

Figure 6: Plasma levels of GnRHa and of GtH in female seabream before and after their treatment with 75:10 PLGA-GnRHa.

Results: The levels of mGnRHa and of GtH in the plasma of the females treated with our PLGA 75:10 microspheres containing the mGnRHa are shown in Fig. 6. As was the case with the PLGA 50:05 (Fig. 5), the PLGA 75:10 released an initial burst of mGnRHa, which peaked 24 hours after the injection of the microspheres. However, this initial burst was much smaller than that observed in the case of the PLGA 50:05, in terms of amplitude and duration of circulating mGnRHa levels (compare Figs. 5 and 6). Following this burst, plasma levels of the mGnRHa decreased to low values, indicating reduced release rates of the mGnRHa from the microspheres. From day 21 on, plasma mGnRHa levels started to rise again, indicating a renewed release of mGnRHa. This probably reflects the initiation of biodegradation. mGnRHa levels were still rising at day 28, the end of the experiment. The pattern of plasma GtH levels was highly synchronous with that of the mGnRHa. The initial burst of the mGnRHa stimulated a transient surge of GtH secretion, GtH levels

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then decreased when the release of the mGnRHa slowed down. Plasma GtH levels increased again when the microspheres again started to release the mGnRHa (Fig. 6). All the treated females ovulated within 72 hours of the administration of the PLGA. However, most of them ovulated daily only during a period of 10-15 days and then stopped. The rest of the vitellogenic oocytes underwent rapid atresia.

Conclusions: The PLGA 75:10 microspheres released a small portion of their GnRHa during the initial 3-6 day period. The major part of the GnRHa is probably released when the polymer starts to undergo biodegradation, at around 3 weeks post administration. The initial low circulating levels of the GnRHa induced intensive, but short-term GtH secretion. Unlike the PLGA 50:05, the initial release of GnRHa was insufficient to maintain elevated rates of GtH secretion over long periods. Therefore, the females treated with the PLGA 75:10 did not ovulate over an extended period of time, and their vitellogenic oocytes became atretic before the GnRHa and GtH levels again increased from the biodegradation of the microspheres. Therefore, it seems that the 75:10 PLGA is not an adequate preparation for the induction of long term daily spawning in seabream. The PLGA 50:05 seems to be a much better candidate for these purpose. However the PLGA 75:10 might be used in cases where GnRHa release over long periods is desired, such as advancement of puberty in salmonids. We will test this possibility in our next year's research.

2.3.1.3. Effect of controlled administration of GnRHa on spawning.

Since the experiments described above show the high efficiency of the 50:05 PLGA-GnRHa microspheres in inducing ovulation and spawning in seabream, we began to upscale this technique for routine use in the induction of commercial spawning in this species (in Eilat). At different times of the year, 20-40 female seabream undergoing the final stages of vitellogenesis were injected with 5005 PLGA-GnRHa microspheres, at a dose of 100-200 μg GnRHa/kg body weight. The females were held in 5-10 m^3 spawning tanks. In most of the cases, spawning started in the tanks containing the treated females as soon as 72 h post treatment, and continued for periods ranging to 3-4 months. The spawned eggs were of a very good quality, with 90-100% fertilization rates and 70-100% hatching rates. In fact, some commercial hatcheries are presently using the 50:05 PLGA-GnRHa systems. for the production of seabream eggs.

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2.3.2. Salmonid species.

2.3.2.1. Effect of controlled administration of mGnRHa on ovulation in rainbow trout.

Methods: This experiment was carried out in a commercial salmonid hatchery in Maine. Three year old maturing female rainbow trout (Donaldson strain) were selected a few weeks before their spawning season. They were divided into 4 groups of 20 each. One group received a single injection of a mGnRHa dissolved in saline (at 20 µg/kg). A second group was implanted with non-coated EVAC devices containing 150 µg of the mGnRHa. A third group was injected with PLGA 50:05 microspheres containing the mGnRHa, at 150 µg/female. The control females received either EVAC devices with no hormone (10 females), or the vehicle solution of the PLGA (10 females). The fish were stocked in 10 m³ fresh-water tanks. At 6, 9, and 30 days after the beginning of the experiment the females were checked for ovulation. Ovulated females were stripped and their eggs were fertilized with freshly collected sperm. Fertilized eggs of each female were incubated in an individual tray, and fertilization and hatching rates were determined for each individual batch.
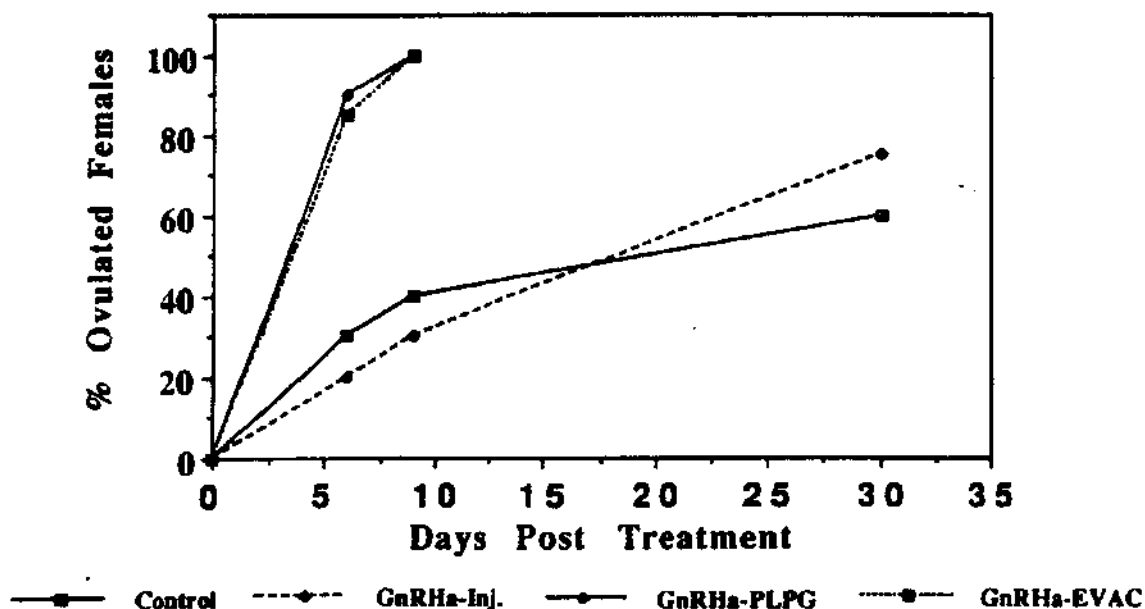

Figure 7: Ovulation rates of female rainbow trout after administering GnRHa by different modes.

Results: The cumulative percent of ovulated female is shown in Fig. 7.
The control females and females given a single GnRHa-saline
injection ovulated in a very non-synchronous manner. Only 50-60%
of these females ovulated by day 30. However, the controlled
administration of the GnRHa, via either the biodegradable PLGA or
the non biodegradable EVAC, induced 100% of ovulations within 9
days. Fertilization and hatching rates of the eggs stripped from the
hormone treated females did not differ significantly from these rates
observed for eggs stripped from the control females. In all cases
fertilization rates ranged from 70-100% and hatching rates from 50-
80%. Similar results on egg quality were obtained in experiments
carried out at the University of Washington at Seattle, on EVAC-
mGnRHa induction of ovulation in trout. The complete analysis of
these experiments will be reported at a later date.

Conclusions: The controlled administration of GnRHa is a much more
efficient method of synchronizing ovulation in rainbow trout than   a
single injection of the hormone. This technique does not affect the
quality of the ovulated eggs. In view of determining the minimum
required dose of GnRHa, further experiments are being carried out to
optimize dosages of the GnRHa and the rates of its release.

2.3.2.2. Effect of controlled administration of mGnRHa on ovulation in
Atlantic salmon.

Methods: This experiment was carried out in a commercial salmonid
hatchery in Maine. Four year old maturing female Atlantic salmon
were selected a few weeks before their spawning season. Twenty
females were treated with uncoated EVAC devices containing 75 µg
of the mGnRHa. Another group of 20 females received similar EVAC
devices which did not contain the GnRHa. Females were stocked in 10
m^3 fresh-water tanks. At 6, 12, 14, 19, and 26 days after the
implantation they were checked for ovulation. Ovulated females
were stripped and the collected eggs were fertilized with freshly
collected sperm. The fertilized eggs of each female were incubated in
an individual tray, and fertilization and hatching rates were
determined for each individual batch.

Results: The results of the present experiment are shown in Fig. 8.
The control females ovulated in a non-synchronous manner, with
only 35% of them having ovulated by day 19 and 75% by day 26. The
controlled administration of the GnRHa via the EVAC induced 50% of
the females to ovulate by day 12 and 100% to ovulate by day 14. No

differences were found in fertilization rates (85-100%) and in hatching rates (50-100%) between eggs obtained precociously from hormonally-treated females and those obtained from the control females.
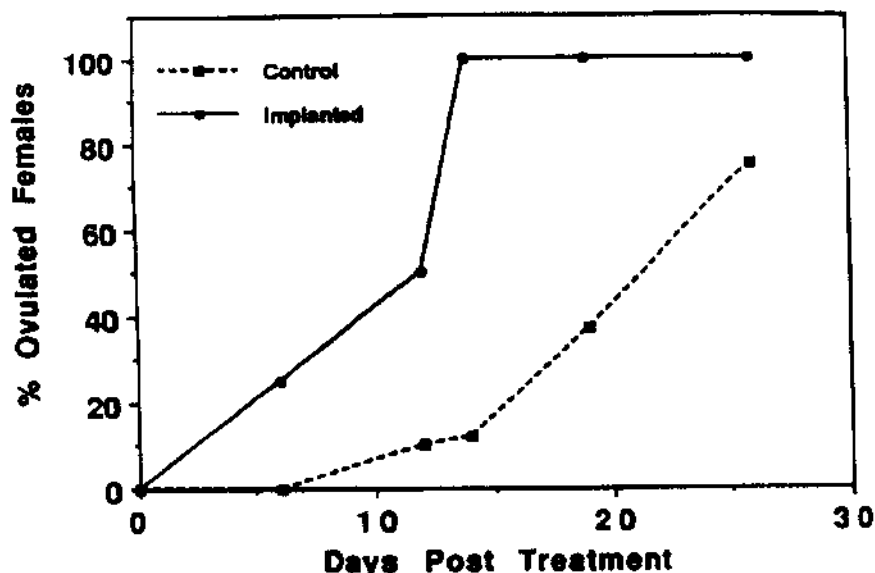

Figure 8: Ovulation rates of female Atlantic salmon after their implantation with EVAC-GnRHa devices.

Conclusions: The controlled administration of relatively low doses of GnRHa via EVAC devices efficiently synchronizes ovulation in farmed Atlantic salmon females. The artificial induction of ovulation does not affect egg quality. On the basis of our first data, we are presently trying to optimize the EVAC-GnRHa preparations, and also the bio-degradable PLGA-GnRHa devices, for their efficient use in the synchronization of spawning in Atlantic salmon. The use of biodegradable devices might be preferred in the case of Atlantic salmon, in which reconditioned spawned females are harvested for human consumption.

2.3.2.3. *In vivo* mGnRHa release from EVAC and its effect on GtH secretion and ovulation in coho salmon.

Methods: The experiments on Pacific coho salmon were carried out in the School of Fisheries, University of Washington at Seattle. Wild coho salmon females running from the sea into a fresh water pond of the university were used. Eighty females were individually tagged and divided into eight groups of 10 each. Four groups were

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implanted with coated EVAC devices. The control group received
devices with no hormone and the three other groups were implanted
with devices containing 25, 75, and 150 μg mGnRHa/device. Four
other groups were implanted with non-coated EVAC devices, with
either no hormone (for the control) or with 25, 75, or 150 μg
mGnRHa/device. The females were checked for ovulation at 7, 9, 11,
13, 16, and 21 days after the implantation. Ovulated eggs were
stripped from the females and fertilized. At all the above times the
females were also bled, and their plasma was immediately frozen
for hormonal analysis. After being bled at the time of ovulation, no
more blood samples were taken from that female. The levels of GtH I
and II were measured in all blood samples, using specific
homologous radioimmunoassays developed by Dr. Dickhoff's group.
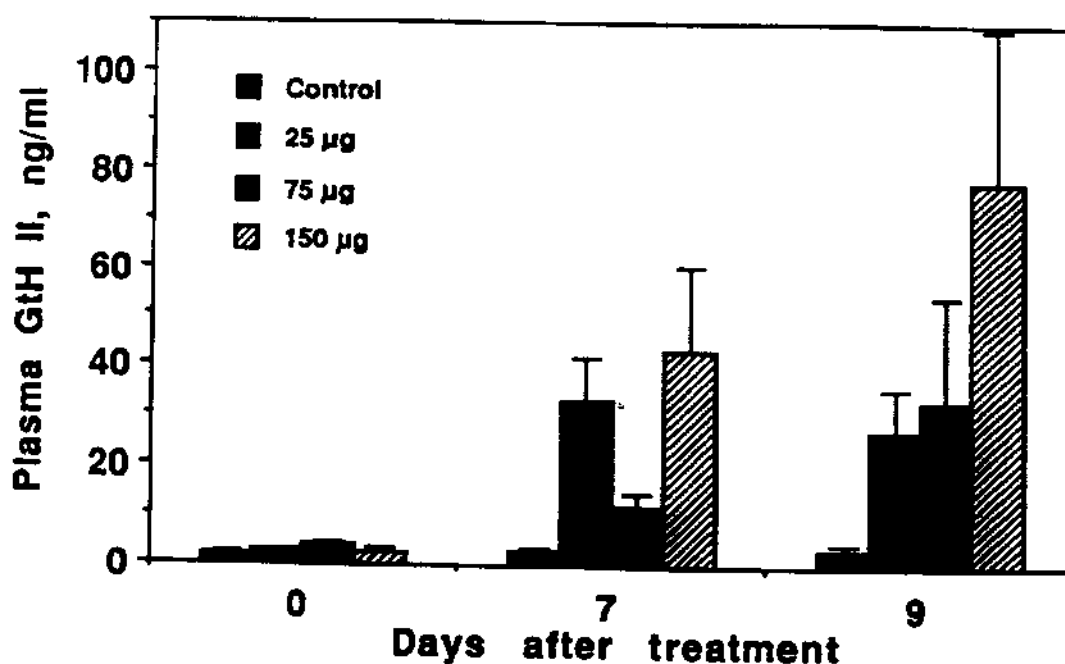Also we measured the levels of the mGnRHa in the blood.

Figure 9: Plasma GtH levels in female coho salmon before and after
their implantation with coated EVAC devices that contain increasing
doses of GnRHa.

Results: The levels of mGnRHa in the plasma of females implanted
with the coated EVAC devices increased significantly by 7 and 9 days
post-implantation (data not shown). These levels were relatively low
(in most cases below 0.5 ng/ml) and we did not observe any
relationships between the mGnRHa dose of the implant and the

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plasma levels of the peptide. These *in-vivo* data are in agreement with the *in-vitro* characteristics of mGnRHa release from coated EVAC devices (see section 2.2.3 and Fig. 1). However, in spite of the very moderate release of GnRHa from the coated devices to the circulation of the treated females, these devices did stimulate GtH secretion. Plasma GtH levels in the implanted females were significantly elevated as soon as 7 days post treatment (Fig. 9). Except for day 9, in which GtH levels in the females treated with 150 μg devices were the highest, we did not observe a relationship between the mGnRHa dose of the device and the plasma GtH levels (Fig. 9). The fact that the coated EVAC devices stimulated GtH secretion resulted in accelerated ovulations (Fig. 10). By day 16 only 50% of the control females ovulated, whereas 100% of the females bearing the coated EVAC-mGnRHa devices ovulated by days 13-16. However, as can be seen in Fig. 9, the treated females did not ovulate in a very synchronous manner.
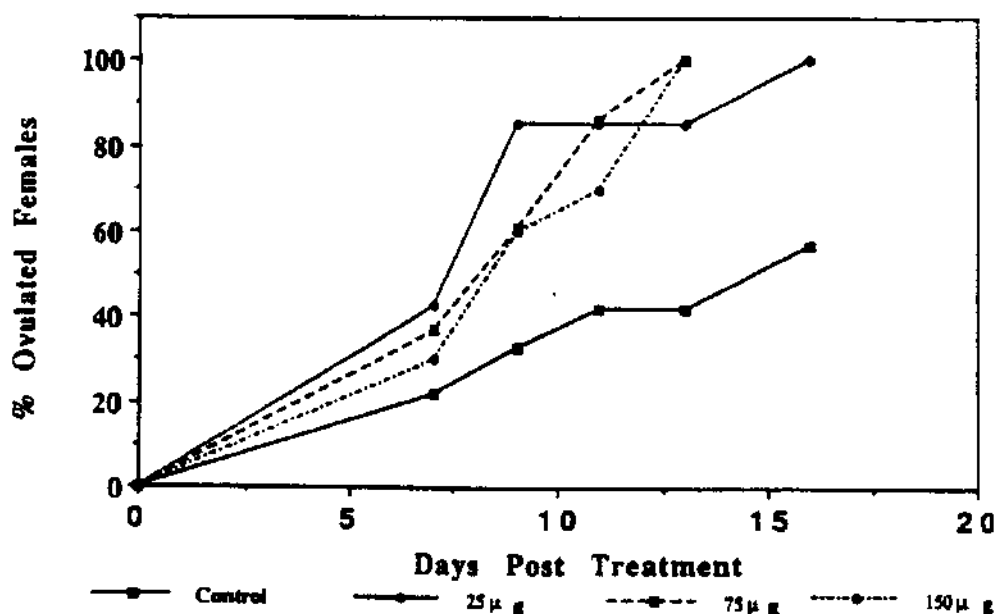

Figure 10: Ovulation rates of female coho salmon after their implantation with coated EVAC devices that contain increasing doses of GnRHa.

Plasma GnRHa levels in females treated with the non-coated EVAC devices are shown in Fig. 11. We can see a very clear dose related release of the mGnRHa from the devices to the plasma of the treated females. At day 7, circulating levels of mGnRHa ranged from
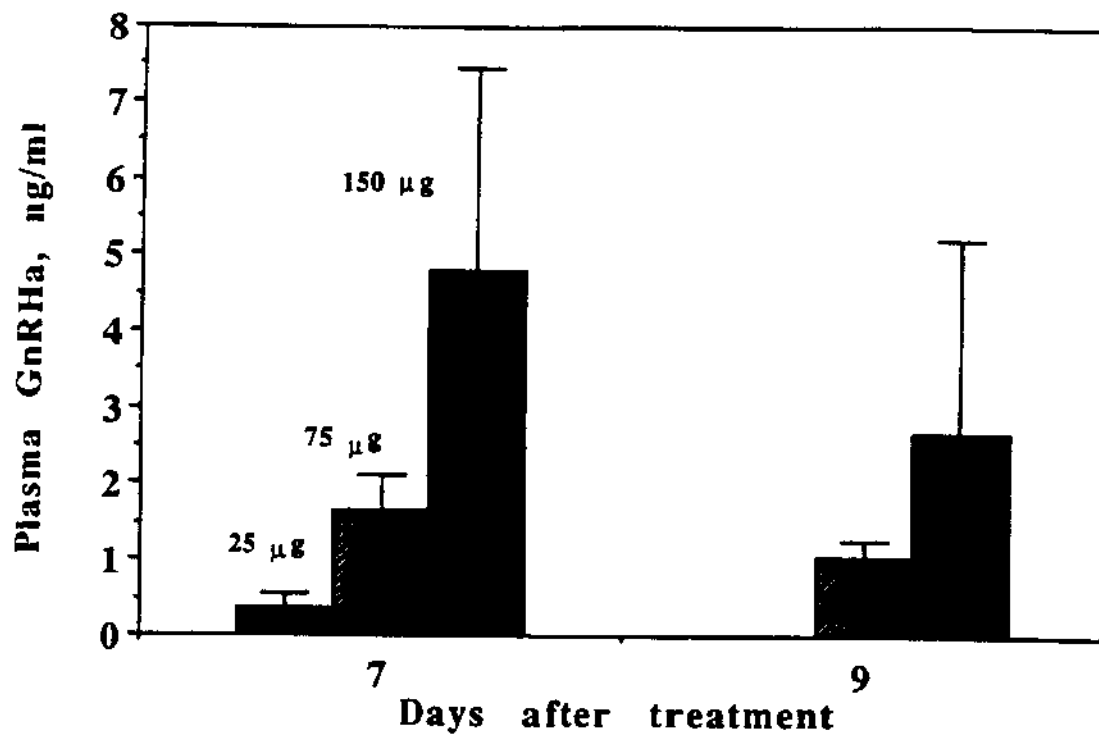
Figure 11: Plasma GnRHa levels in female coho salmon after their implantation with uncoated EVAC devices that contain increasing doses of GnRHa
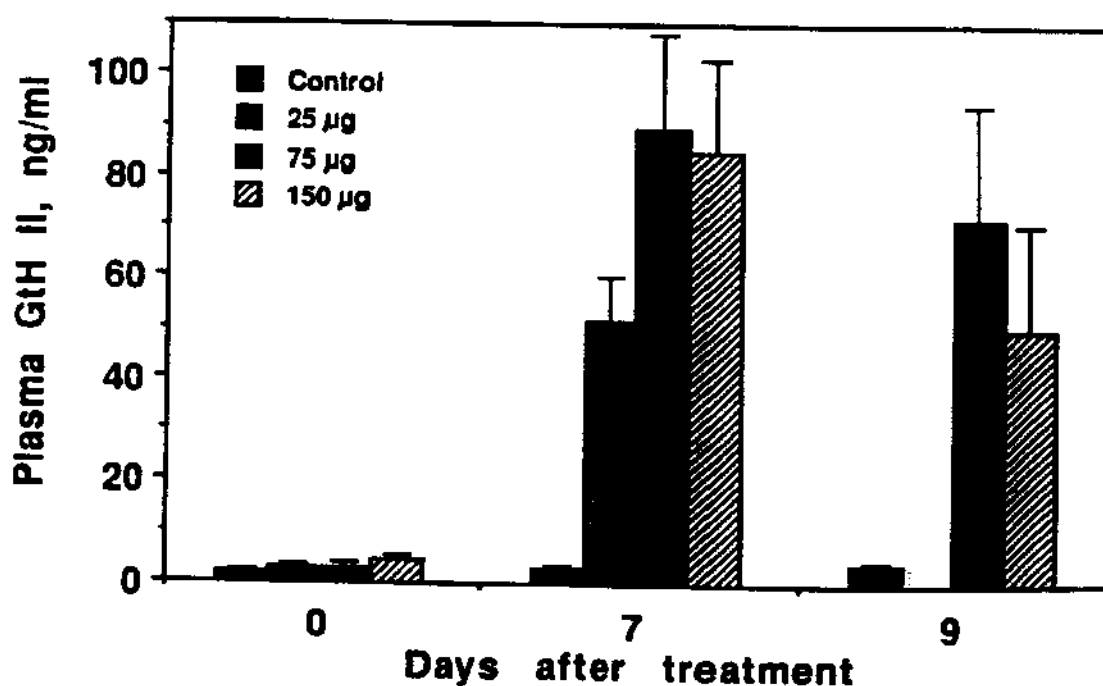

Figure 12: Plasma GtH levels in female coho salmon before and after their implantation with uncoated EVAC devices that contain increasing doses of GnRHa.

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around 0.5 ng/ml in females treated with the 25 μg devices to 4.8 ng/ml in females bearing the 150 μg devices. These relationships are partially reflected by the pattern of plasma GtH levels in the same females (Fig. 12). At day 7 after the implantation, plasma GtH levels in females bearing the 75 and 150 μg devices were significantly higher than those found in the females treated with the 25 μg devices. After day 7, the release rate of the mGnRHa from the non-coated devices slowed down (Fig 11). The *in-vivo* release pattern of mGnRHa from uncoated EVAC devices in coho salmon is in a very close agreement with the *in vitro* release patterns of the peptide from the same devices at the same temperature (see section 2.2.3 and Fig. 2).
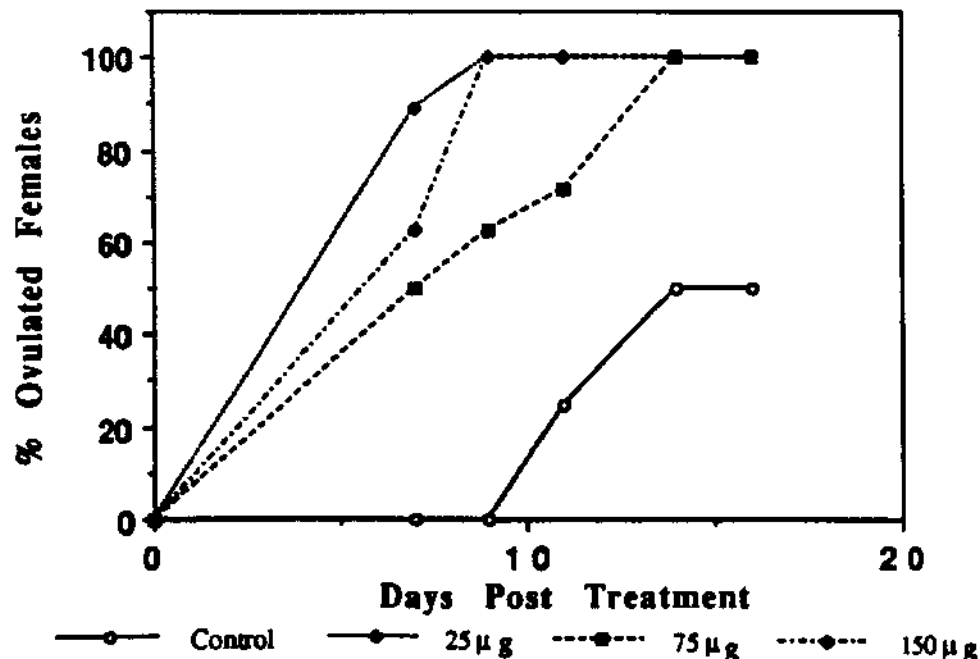

Figure 13: Ovulation rates of female coho salmon after their implantation with uncoated EVAC devices that contain increasing doses of GnRHa.

When comparing the *in vivo* hormonal data obtained with the two different types of implants, it is very clear that the uncoated EVAC devices released the mGnRHa to the blood much faster than the coated ones, and that this more intensive release was reflected by a much stronger stimulation of GtH secretion in the females treated with the uncoated EVAC devices (compare Figs 9 and 12). These

differences are also reflected in the potential of the two types of devices to synchronize ovulation. The uncoated EVAC-mGnRHa devices were found to be much more efficient in synchronizing ovulation (Fig 13) than the coated ones (Fig. 10). Where only 50% of the control females ovulated by day 16, all the females treated with the uncoated EVAC-mGnRHa devices ovulated within a period of 9 days (for the 25 and 150 µg devices) to 13 days(for the 150 µg devices) post implantation (Fig. 13), which is a very good synchronization of ovulation.

Conclusions: The EVAC devices were found to efficiently release mGnRHa to the blood of female coho salmon and to be very potent in inducing GtH secretion and ovulation. The uncoated devices released their mGnRHa content much faster than the coated devices. In agreement with our *in-vitro* release data, the uncoated devices released most of their mGnRHa within a period of 7 days. The intensive release of the mGnRHa from the uncoated devices results in a stronger stimulation of GtH secretion and in a more efficient synchronization of ovulation than that achieved with the coated devices. It is concluded that uncoated EVAC devices containing as low as 25 µg mGnRHa are highly effective in synchronizing ovulation in coho salmon. This conclusion is of a major applied importance in regard to the manipulation of spawning in coho-salmon farming. We are presently continuing our studies on the use of controlled release of GnRH analogs for the manipulation of spawning in a few species of Pacific salmonids.

3. GENERAL CONCLUSIONS

During the first two years of our research we produced different polymeric controlled release delivery systems containing various super active analogs of GnRH. Both bioerodible (PLGA) and controlled diffusion (EVAC) systems were made. We started to characterize the *in vitro* and *in vivo* release patterns of the GnRH analogs from the different delivery systems. The release rates of GnRHa from the different devices into the blood of seabream and Pacific salmon were studied, both in relation to their stimulation of GtH secretion and to their induction of ovulation and spawning. Controlled administration of GnRH analogs via some bioerodible and non bioerodible polymeric devices was found to be highly effective in inducing successful spawning in a variety of commercially important farmed species, including seabream, rainbow trout and

Atlantic and Pacific salmon. We are presently continuing our research on the optimization of the above technology, according to our proposed research plan.